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Tube fusion: making connections in branched tubular networks

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Summary

Organs like the vertebrate vascular system and the insect tracheal system develop from separate primordia that undergo fusion events to form interconnected tubular networks. Although the correct pattern of tubular connections (anastomoses) in these organs is crucial for their normal function, the cellular and molecular mechanisms that govern tube fusion are only beginning to be understood. The process of tube fusion involves tip cell specification, cell-cell recognition and contact formation, self-avoidance, changes in cell shape and topology, lumen formation, and luminal membrane fusion. Significant insights into the underlying cellular machinery have been gained from genetic studies of tracheal tube fusion in *Drosophila*. Here, we summarize these findings and we highlight similarities and differences between tube fusion processes in the *Drosophila* tracheae and in the vertebrate vascular system. We integrate the findings from studies *in vivo* with the important mechanistic insights that have been gained from the analysis of tubulogenesis in cultured cells to propose a mechanistic model of tube fusion, aspects of which are likely to apply to diverse organs and organisms.

Contents

1. Introduction

Tubular organs, such as the lungs, mammary glands, kidneys, and the vascular system perform vital body functions, such as gas exchange, excretion, and nutrient transport. The cellular and molecular principles underlying branching morphogenesis in these organs are being revealed by many studies in vertebrate and invertebrate model systems. Developmental programs involving reiterative rounds of spatiotemporally controlled branching events may explain the complex structures of organs such as the mammalian lungs [1]. An additional layer of complexity is found in organs such as the vascular system, where vessels link up with each other to form anastomoses (connections between branches), thus giving rise to interconnected tubular networks [2-4]. Using terms from network theory, lungs and mammary glands represent tree-like structures, whereas the vascular system represents a mesh-like structure. Constructing a mesh-like network requires, in addition to branching events, the formation of connections between specific branches in the network. In biological terms, this involves the recognition and formation of contacts between specific cells, followed by morphogenetic events that generate a patent lumen for efficient liquid transport through the tubular connection. This process, which we refer to as tube fusion, takes place in organs, which derive from initially separate tubular units that get connected during subsequent development. During angiogenesis, the vascular network is remodelled through the fusion of certain endothelial sprouts with each other or with pre-existing vessels [5, 6]. Despite the fundamental role of this process in angiogenesis, only few studies have investigated the mechanism of blood vessel fusion [5, 7-10]. Notably, live imaging studies in zebrafish have revealed a first view on blood vessel fusion at cellular resolution [5, 7, 9]. Besides angiogenesis, tube fusion also occurs during vertebrate kidney development. The kidney originates from separate precursors, the metanephric mesenchyme and the ureteric bud, each of which form tubes that subsequently fuse to generate a functional excretory system [11, 12]. Despite their key role in nephron formation, the cellular and molecular basis of these fusion events is not understood. Tubular networks are also found in invertebrates. The insect tracheal system is a network of gas-filled epithelial tubes that delivers oxygen to the tissues (Fig. 1A; [13-16]). Tracheal development shares considerable similarities with angiogenesis: in both cases branched tubular trees are built through tip-cell-guided collective cell migration, and tube fusion events generate anastomoses between branches in the tree (Fig. 1F,G; [7, 17]). Genetic studies of tracheal tube fusion in *Drosophila* have revealed insights into the underlying cellular and molecular machinery. In this review, we summarize these findings, highlighting similarities and differences between flies and vertebrates.

2. Tube fusion: what has to be accomplished

The formation of connections in epithelial or endothelial tubular networks is orchestrated by specialized cells located at the tips of the branches undergoing fusion. Tip cells were first described in the developing vascular system of quails and in the tracheal system of *Drosophila melanogaster* nearly 20 years ago [18, 19], although their significance in leading angiogenic sprouts was only demonstrated by studies of angiogenesis in the mouse retina some years later [20]. In the following, we first discuss the mechanisms of tip cell selection and of tip cell-guided migration of fusion sprouts towards each other. Next, we review the mechanisms of cell-cell recognition, contact formation, and repolarization involved in tube fusion. Finally, we discuss roles of the cytoskeleton and of the membrane trafficking machinery in generating, expanding and joining luminal spaces. In this context, it is important to emphasize that tip cells actually do not fuse during tube fusion, unlike, for instance, myoblasts, which form syncytia during muscle development. Instead, the term tube fusion [21] refers to a cell hollowing mechanism employed by tip cells to connect ("fuse") pre-existing lumina formed by the stalk cells (SCs) adjacent to the tip cells.

3. The *Drosophila* tracheal system as a model for studying tube fusion

The tracheal tree develops from twenty epidermal placodes, ten on each side of the embryo, each composed of approximately 80 cells, which invaginate and thereby create a lumen facing the apical surface of tracheal cells. From each such invagination groups of cells migrate out in a stereotyped pattern to form primary branches (Fig. 1A; [19]). Certain primary branches emerging from each tracheal primordium connect with cognate branches from adjacent (lateral and contralateral) primordia to build an interconnected network with a continuous lumen that permeates the entire system. These tubular connections, or anastomoses, are generated in stereotyped positions between neighbouring axial branches and between contralateral dorsal branches that migrate towards the midline in each tracheal metamere (Fig. 1B,E; [21]). Anastomosis formation is accomplished by specialized tip cells called fusion cells (FCs), which recognize, migrate towards, and connect with a specific partner FC (Fig. 1B,C). A second type of specialized tip cells called terminal cells (TCs) forms lumenized cytoplasmic extensions that contact the target tissues and mediate gas exchange (Fig. 1B,D). The spatial distribution of the two different tip cell types is stereotyped and essential for the final architecture and function of the tracheal system. Intriguingly, FCs and TCs form intracellular (seamless) tubes, unlike all other tracheal cells, which enclose extracellular lumina sealed by cell-cell junctions [22-24].

In the following, we go step-by-step through the tracheal tube fusion process, with a focus on events happening in the FCs (Fig. 2).

4. Steps of the tube fusion process

4.1. How to get to the right place: tip cells lead branch migration

The sprouting of tracheal branches is controlled by the chemoattractant Branchless (Bnl), a Fibroblast Growth Factor (FGF) protein produced by small clusters of ectodermal and mesodermal cells surrounding each tracheal placode [25]. Bnl activates the FGF receptor (FGFR) homolog Breathless (Btl; [25, 26]), which is expressed under the control of the bHLH-PAS transcription factor Trachealess (Trh) in tracheal cells [27]. Cells at the tip of each sprouting branch respond to the Bnl stimulus by MAPK/ERK activation [28], by the expression of genes including the ETS domain transcription factor Pointed (Pnt; [19]) and the negative feedback regulator Sprouty (Sty; [29]), and by the extension of actin-based filopodia [30]. How FGF signalling acts to guide tracheal cell migration is still not clear. Interestingly, MAPK activation is not sufficient to rescue the migration defect of *btl* mutants, suggesting that another branch of the pathway, probably not relying on transcriptional regulation, controls the machinery necessary for migration [30]. Tip cells have elevated levels of activated Rac GTPase, which promotes the formation of cellular protrusions [31]. This and the finding that cells with impaired Rac activity rarely occupy the leading position suggests that the sustained and localized stimulation of the Btl receptor leads to the activation of small GTPases, which in turn induce cytoskeletal changes resulting in directional migration [31]. Tip cell motility is necessary and sufficient to support the movement of all trailing (stalk) cells of the branch [32]. However, tip ("leader") cell role is not predetermined or a fixed state of a given cell in the branch. Instead, leadership strictly depends on the level of Btl/FGFR receptor activation. Supporting this notion, cells lacking a functional Btl receptor surrounded by wild-type tracheal cells never occupy the tip position. Conversely, single cells with wild-type Btl levels often locate to branch tips in a *btl* heterozygous tissue environment [33] and can rescue the migration defects of *btl* mutants [31]. Furthermore, leadership is a dynamic role, because upon laser ablation a tip cell is readily substituted by one of the trailing SCs [32]. Remarkably, tracheal branch outgrowth shares many similarities with angiogenesis, where vessel sprouting is guided by Vascular Endothelial Growth Factor (VEGF) signalling. VEGF triggers MAPK activation in endothelial cells and stimulates their motility and proliferation [2, 20]. Like in tracheal morphogenesis, endothelial cells closest to the VEGF source and with strongest VEGF Receptor 2 (VEGFR2) activation dynamically compete for the leading position [4, 34].

4.2. Assigning the job: fusion cell specification

Anastomosis formation has to be spatially controlled, such that connections are built only in the correct places. This may be achieved by spatial constraints from the surrounding tissues, which restrict the formation of anastomoses to certain positions, or by making only a selected set of tip cells competent to form anastomoses. In the tracheal system, the latter scenario is the case. Tracheal tip cells differentiate into either of two specialized cell types, FCs or TCs, which are specified by different inducing signals, express different sets of genes, and adopt distinct morphologies (Fig. 1A,C,D). Initially all tracheal tip cells express the MAPK target genes *pnt* and *sty* [19]. Expression of these genes subsequently becomes restricted to the future TCs, where Pnt regulates other genes such as DSRF, which promote lumen growth [19, 35]. FC fate is specified by local Wg/WNT and Dpp/TGFβ signals produced by surrounding cells [36-38]. These signals are sufficient to induce all genes necessary for tube fusion, also when the signalling pathways are induced ectopically in tracheal non-tip cells. Wg-Armadillo/β-Catenin signalling induces the expression of effectors of the tube fusion program, but also of genes that prevent neighbouring cells from adopting FC fate. A key transcription factor expressed in all FCs is the Zinc finger protein Escargot (Esg). Esg is required for upregulating E-Cadherin (E-Cad) expression and for repressing TC genes in FCs [21, 39]. An indirect Esg target gene is the bHLH-PAS transcription factor Dysfusion (Dys; [40, 41]). Dys is required for FC-specific gene expression and for downregulation of *trh* (and consequently *btl*) in FCs [40-42]. Although misexpression of Dys is sufficient to induce ectopic branch fusions, these ectopic connections do not form lumina, suggesting that Dys target genes mediate early steps of the fusion program, but are not sufficient for lumen formation [41]. FCs also specifically express the Notch ligand Delta (Dl; [38]). Dl activates Notch signalling in neighbouring SCs, thus preventing them from becoming FCs via lateral inhibition. Consequently, Notch mutations lead to excessive FC specification [36, 43, 44]. Similarly, endothelial tip cells express the Delta homologue Dll4 and inhibit neighbouring SCs from becoming tip cells through Notch signalling. Notch inhibits VEGFR2 expression in SCs, thereby blocking tip cell fate [45-48]. Conversely, Notch promotes expression of a second receptor, VEGFR1, which has low kinase activity and acts as a sink for VEGF [34]. Thus, Notch-dependent differential regulation of two VEGF receptors controls endothelial tip cell selection. Furthermore, Notch activation in angiogenic tip cells is kept lower than in SCs through a mechanism mediated by Jagged1, another Notch ligand expressed at elevated levels in SCs [49]. While the roles of Notch signalling in angiogenic tip cell selection have been extensively investigated (for up-to-date reviews see [2, 4] and review by Schuermann *et al.* in this issue), the molecular effects of Notch signalling in tracheal SCs are not clear, and neither is the role of Dl in

FCs. It was shown, however, that the mere restoration of Dl signalling in the absence of Wg signalling is not sufficient to rescue FC specification, suggesting that other effectors of Wg are involved [38]. Besides Notch signalling, an additional mechanism prevents tracheal SCs from acquiring FC or TC cell fate. Anterior Open (Aop), an ETS-domain transcriptional repressor that antagonizes Pnt [50], is expressed in all tracheal cells, but is degraded upon MAPK-mediated phosphorylation in tip cells [29]. Loss of Aop leads to excessive misspecification both of FCs and TCs [51]. This was explained by a dual mode of Aop function: Aop inhibits TC fate through antagonizing Pnt [29], while at the same time it inhibits FC fate by antagonizing Wg signalling [51]. Intriguingly, recent work showed a role for the vertebrate Aop homologue Tel/Etv6 in angiogenesis. Here, Tel represses genes required for sprouting, including Dll4 and Sprouty4, a homologue of *Drosophila* Sty [52]. Thus, Notch signalling and Aop-mediated repression are two essential mechanisms for restricting tip cell fate both in tracheal development and angiogenesis. However, it is not clear in either system whether Notch and Aop act separately, increasing the robustness of tip cell selection, or whether these pathways cross-talk.

In the light of these striking molecular similarities between tip cell selection in angiogenesis and tracheal development, some important differences should be emphasized. First, in the vascular system all tip cells, once selected as such, are capable of forming anastomoses. In contrast, tracheal FCs are one of two alternative tip cell types whose precise positioning determines where anastomoses are built. Second, vascular tip cells can form anastomoses not only with other tip cells, but also with cells of non-sprouting vessels. In addition, vessel sprouts and anastomoses can dynamically extend or regress. Blood flow and shear forces are increasingly recognized as key factors in controlling angiogenic sprout outgrowth, regression, and pruning [53-56], and these physical cues are likely to influence also the formation, retraction and maintenance of anastomoses.

4.3. Finding the right partner: cell-cell recognition, self-avoidance, and contact formation

Once FCs have been specified and are migrating, their next tasks include recognition of the correct partner cell and formation of a stable cell-cell contact, followed by the arrest of migration and the establishment of polarity along the future tube axis (Fig. 2A,B). While the Bnl/Btl system is essential for tracheal cell migration, Bnl is probably no longer involved as a chemoattractant once the FCs have come in close vicinity, because *btl* expression is downregulated in FCs [41]. Instead, FCs might mutually attract each other via short-range signals (e.g., chemokines). However, a chemokine signalling mechanism must avoid self-stimulation and therefore cannot readily explain mutual attraction, unless the two partner cells use different chemokines and complementary receptors. A

fascinating solution to this problem was described in vegetative hyphal fusion in the Ascomycete *Neurospora crassa* [57]. Here, genetically identical cells, as in case of tracheal FCs, are able to sense each other in close proximity by communicating via chemoattractants. Each of the two approaching hyphal cells recruits either one of two proteins to the plasma membrane in an oscillating fashion. Through these oscillations, cells rapidly alternate between states of a chemokine sender and receiver. Thereby, each of the two cells not only stimulates directed growth of the partner, but also avoids self-stimulation by the signal [57]. It is conceivable that similar systems could be used for cell-cell recognition during organogenesis in multicellular organisms.

An alternative solution to the mutual attraction problem could be provided by additional cells acting as a "bridge" for the approaching pair of FCs, which would adhere to the bridge cell before contacting each other. Evidence for this scenario has been reported in the tracheal system, as well as in angiogenesis. It was shown that direct contact between angiogenic tip cells and macrophages supports efficient anastomosis formation in mice and zebrafish, although the analysis of macrophage-deficient mice revealed that macrophages are not essential for vessel sprouting [8]. Similarly, in *Drosophila* single mesodermal "bridge" cells are located between each pair of FCs in the tracheal dorsal trunk (DT). FCs directly contact the bridge cells, which are required for DT fusion [58, 59]. It was subsequently shown that the bridge cells produce Bnl, which is essential for the initial migration of DT cells [60]. However, Bnl expression is not sufficient for bridge cell function in tube fusion [61]. Instead, the adhesive properties of bridge cells appear to be critical. Capricious, a leucine-rich repeat (LRR)-containing adhesion protein, is expressed selectively in bridge cells [61]. Genetic experiments suggested that Capricious acts instructively as a guidance cue for FCs, while another LRR protein, Tartan, may provide a permissive substrate for tracheal cell migration.

Once in close proximity, partner FCs make their first contact via long filopodia projected in the direction of migration [21, 39, 62]. The same behaviour was described for angiogenic tip cells undergoing anastomosis in zebrafish embryos [5, 7]. Surprisingly, however, recent work showed that filopodia are dispensable for endothelial migration and tip cell guidance, but are important for anastomosis formation [10]. Tracheal FCs make stable contacts only with another FC, but not with other tracheal cell types, even in case of occasional erroneous fusion events between two distant FCs [51]; own unpublished observations). This suggests that FC filopodia carry adhesion molecules that are expressed specifically in FCs. However, while a FC has to recognize its partner among other cells, it also has to avoid making contacts with itself as its numerous filopodia are searching for a

partner. The problem of self-avoidance has been extensively studied in neurons, which develop arborized structures with many cellular processes that have the capacity to avoid each other if they emerge from the same cell (reviewed in [63]). This property is mediated by cell surface receptors, Dscam in *Drosophila* and Protocadherins in vertebrates, which exist in many (ten thousands in case of Dscam) distinct isoforms. These isoforms have different extracellular parts but share the same intracellular part, which triggers a repulsive output when compatible isoforms bind to each other. Although candidate receptors such as Dscam appear not to be expressed in the trachea, FCs could in principle employ an analogous receptor-matching-based mechanism for self-avoidance.

Once partner FCs have made contact via filopodia, this contact is stabilized through homophilic adhesion. E-Cad plays a key role here and is transcriptionally upregulated in FCs under the control of Esg and Dys [39, 41]. Reduction or loss of E-Cad in *Drosophila*, or of VE-Cadherin (VE-Cad) in vertebrates, leads to anastomosis defects [9, 39, 64-66]. E-Cad in tracheal and VE-Cad in endothelial cells play important roles in organizing the cytoskeleton and in establishing and maintaining cellular polarity. In migrating tracheal FCs, an E-Cad ring seals the apical surface facing the adjacent SC lumen. As soon as the migrating partner FCs have met, a focal accumulation of E-Cad appears at the contact point opposite to the FC-SC junction (Fig. 2B). This E-Cad spot subsequently expands into a ring-shaped adherens junction (AJ) between the two FCs [39, 62]. In addition, the apical polarity complex (Bazooka/Par3, aPKC, Crumbs) and the apical proteins Stranded at second (Sas) and Discs Lost/PATJ accumulate at the contact point, thus defining a second apical domain and rendering the FCs bipolar (Fig 2C, Fig. 3; [62, 67]). It was proposed that E-Cad directly or indirectly recruits apical determinants to the newly forming apical domain [62, 67]. This hypothesis was challenged by a recent report in which the behaviour of zebrafish endothelial tip cells lacking VE-Cad was carefully analysed [9]. Strikingly, the localization of VE-Cad in anastomosing endothelial cells resembles the dynamic redistribution of E-Cad from an apical spot into a ring in *Drosophila* FCs. Surprisingly, tip cells lacking VE-Cad still made contacts and delivered apical determinants to the contact site. However, instead of making a single stable contact, multiple contacts were made and tip cells continued their sprouting activity, suggesting that cells failed to recognize that contacts have been established with a partner cell. These findings show that VE-Cad is not essential for contact formation or to initiate repolarization, and suggest that the adhesion molecules that mediate specific contact formation remain to be identified.

4.4. Pushing and pulling: force generation in fusion cells

FCs dramatically change their shape and cytoskeletal organization during tube fusion. Once FCs have become bipolar with apical domains and AJs present at opposite sides of the cell, the cytoskeleton starts to remodel (Fig. 2C). First, F-actin accumulates at the FC-FC contact, forming a second apical cortex. Subsequently, the two apical cortices get connected by a compact F-actin track, which spans the entire FC and anticipates the future axis of the lumen [67, 68]. Microtubules (MTs), initially enriched below the apical cortex [67, 69], are reorganized into compact bundles that are deposited along the actin track and contain the plus-end-binding proteins EB1 and Clip190 concentrated near the apical membranes [68]. The AJ components E-Cad and β-Catenin were shown to be necessary for assembling the actin/MT track. E-Cad can recruit actin and MTs to the track, and both actin and MTs are required for the maturation and stability of this structure [68]. In addition, the plakin Short stop (Shot) is involved in building the cytoskeletal track (Fig. 3; [67]). Shot is a large bi-functional protein containing actin- and MT-binding domains that localizes to AJs and transiently at the actin/MT track. In *shot* mutant FCs both actin and MTs are disorganized and no track is formed, demonstrating Shot's important role in shaping the track and connecting it to AJs. Another class of cytoskeletal regulators, the Formins, nucleate long and unbranched actin fibres in structures such as filopodia and the contractile ring formed during cytokinesis (reviewed in [70]). Several studies indicate that Formins can also promote microtubule organization along with actin (reviewed in [71]). Interestingly, one Formin family member, Formin3, is specifically expressed in tracheal cells [72]. *formin3* mutations specifically affect the cytoskeletal track in FCs: cortical F-actin and filopodia are normal in *formin3* mutant tracheal cells. However, in FCs the actin track is absent, although actin still accumulates at FC contact points [72].

The actin/MT-based central track is likely to serve special functions related to FC morphogenesis. It may provide a highway for transporting vesicles and organelles by MT-dependent motor proteins. Similarly specialized organization of MTs is found in cells where trafficking needs to be focused towards specific locations. Examples are the antiparallel MT bundles organized by the central spindle in cytokinesis, which deliver membrane material for the fusion event during abscission, the parallel MT bundles that organize cilia, and the MT network that focuses cytotoxic granule exocytosis to the immunological synapse in lymphocytes (reviewed in [73]). In all of these examples centrosomes are positioned to nucleate MTs in a spatially controlled fashion. In contrast, it is not clear how and where microtubules are nucleated in tracheal FCs. MT nucleation in the apical cortex of tracheal cells was shown to be independent of centrosomes, but to depend on the Zona Pellucida (ZP) transmembrane protein Piopio [69]. ZP proteins support mechanical linkage between the

plasma membrane-associated cytoskeleton and the extracellular matrix in epithelia [74, 75]. Intriguingly, one ZP protein, CG13196, is specifically expressed in tracheal FCs, where it might be involved in organizing MTs, although its function is not known [41, 76].

In addition to roles in vesicle transport, the actin/MT track could mediate cell shape changes. After contact formation FCs start to contract, while the adjacent SCs elongate and invade the FCs, resembling a “finger poking into a balloon” (Fig. 2G). This unusual configuration was first observed by Uv and colleagues in electron micrographs [16] and was subsequently corroborated by single-cell labelling experiments [62]. As fusion proceeds, the invading SC tips move closer to the FC-FC contact point (Fig. 2F). Evidently, mechanical force has to be generated to either push or pull the two FC-SC junctions closer together. The nature of the underlying forces is not clear. In principle, SCs could generate pushing forces, while FCs could generate pulling forces. The invading SC could use its assembly of stable apical microtubules parallel to the luminal axis to propel its junction with the FC forward, while the actin/MT track inside the FC could contract and thereby pull on the FC-SC and FC-FC junctions. Contractile forces in FCs could be generated by sliding of actin filaments promoted by Myosin, which is present in the FC track [72], reminiscent of the contractile actin ring in cytokinesis [77]. However, MTs may also generate pulling forces, as in case of mitotic spindle positioning [78, 79]. The important role of MTs in FCs is underlined by the finding that overexpression of the MT severing protein Spastin in tracheal cells inhibits tube fusion [62].

Additionally, forces generated in the extracellular space are likely to contribute to lumen morphogenesis. Blood pressure was reported to be essential for pushing the apical membranes of anastomosing tip cells towards the fusion point [9]. In *Drosophila*, the apical extracellular matrix of expanding tracheal tubes contains secreted proteins and a scaffold of chitin fibres, which are crucial for shaping the luminal membrane (reviewed in [80]). Luminal hydrostatic pressure and mechanical forces imposed by the chitin scaffold on the FC apical surface could also contribute to pushing the FC membrane inwards.

4.5. Cell hollowing: generating and connecting luminal spaces

In the final stage of tube fusion the two SC lumina invading the FCs have to be joined to generate a continuous transcellular lumen that penetrates the FCs. This is accomplished by cell hollowing, which transforms the FCs into toroidal cells surrounding a seamless lumen (Fig. 2D,E). Cell hollowing also occurs in blood vessel anastomosis formation in vertebrates, although there it is not the only mechanism employed to join lumina. In contrast to tracheal FCs, endothelial tip cells are able to connect luminal spaces between fusing tubes also by undergoing extensive junctional

remodelling, thus rearranging into a multicellular configuration [7, 9]. A remarkably similar mechanism involving the connection of separate luminal pockets through extensive cell rearrangements was described to mediate lumen formation in the notochord of the Ascidian *Ciona intestinalis* [81].

FC hollowing can be divided into two steps: lumen initiation and lumen connection (Fig. 3). Fundamental insights into the process of lumen initiation have been gained from studies of tubulogenesis in Madin-Darby Canine Kidney (MDCK) cells, which form lumenized cysts in 3-D culture (reviewed in [82]). FCs, like MDCK cells, deposit apical membrane and luminal material at the FC-FC contact, where an extracellular luminal space is generated *de novo* (Fig. 3; [62, 83]). In MDCK cell cysts and likewise in tracheal cells, establishment of a new apical domain is a prerequisite for lumen formation. At cell-cell contacts of early MDCK cysts, polarity proteins, including Par3/Bazooka and aPKC, accumulate at the Apical Membrane Initiation Site (AMIS; [84]). This platform for apical membrane delivery is marked not only by proteins, but also by the asymmetric distribution of phospholipids, in this case phosphoinositol 2 phosphate (PIP2; [85]). Direct or indirect interaction with PIP2 is crucial for the recruitment of small GTPases such as Cdc42, and of exocyst and SNARE complex components to the AMIS [84, 86]. Upon apical membrane delivery, the AMIS is transformed into the Pre-Apical-Patch (PAP). Apical proteins including Crumbs and Podocalyxin start to accumulate at the PAP, and aPKC and Bazooka are pushed from the PAP to the junctional region [84, 87]. Notably, a similar distribution of Bazooka and apical proteins was observed during lumen formation both in tracheal FCs and TCs [22, 23, 62]. In MDCK cells apical components are endocytosed from the basolateral surface and are then transported in Rab11-positive recycling endosomes to the subapical region. There they are loaded with other Rab proteins (Rab3, 8 and 27), which interact via Synaptotagmin-like proteins with exocyst and SNARE complex components, which in turn guide the tethering and fusion of the vesicles with the correct plasma membrane domain [84, 86]. Various mechanisms contribute to the expansion of the newly formed lumen [82]. In contrast to MDCK cells, expansion of the FC central lumen is only observed in mutant conditions that prevent fusion of the central lumen with the SC lumina [62, 83]. Normally the FC central lumen, once initiated, is rapidly connected with the invading SC lumina.

In order to generate a patent lumen, four apical plasma membranes (two per FC) need to fuse. This could be achieved either through direct plasma membrane fusion, or it could be facilitated by an intermediary membrane compartment that bridges the gap between the two invading SC lumina (Fig. 3). Based on the available data neither of these possibilities can be ruled out. It was proposed that

FCs may form an intracellular lumen by targeting vesicles to the central actin/MT track, where the vesicles would coalesce, possibly generating an expanding membrane compartment (Fig. 3; [21, 67, 83]). Similarly, vacuole-like compartments were observed in endothelial cells and were suggested to contribute to lumen formation and apical membrane delivery in sprouting blood vessels [66, 88]. However, observing vesicle trafficking in tracheal FCs at adequate resolution *in vivo* has been challenging, and the origin of the membrane material that forms the FC lumen is not yet clear. An informative finding was that the exocyst subunit Sec5 is required for tracheal tube fusion [83, 89]. The exocyst is an octameric protein complex, which tethers secretory vesicles at the plasma membrane and spatially controls vesicle fusion (reviewed in [90]). Sec5 is enriched at FC apical membranes and on vesicles along the cytoskeletal track. Some of these vesicles contain the recycling endosome markers Rab11 [89], suggesting that the exocyst and recycling endosomes participate in generating the FC lumen (Fig. 3). Moreover, the small GTPase ARF-like 3 (Arl3) encoded by the *dead end* (*dnd*) gene was shown to be required for lumen fusion [83, 89]. Loss of Arl3, which is expressed exclusively in FCs, leads to the loss of Sec5 accumulation at the FC-FC contact point and along the actin/MT track. The exact molecular role of Arl3 is not clear, but it was shown that Arl3 can interact with MTs, suggesting that Arl3 could mediate the association of Sec5-positive vesicles with the actin/MT track [83]. Since constitutive Arl3 activation promotes actin depolymerisation it was proposed that Arl3 might also help to disassemble the cytoskeletal track during late stages of fusion [89].

In the proposed models of lumen connection the actin/MT track spanning the FC cytoplasm provides a target for membrane delivery, resembling the function of the midbody in targeting membrane traffic to the abscission site during cytokinesis in mammalian cells [77]. The hypothesized intracellular membrane compartment in FCs might function like the cell plate in plant cells, which fuses with the plasma membrane during abscission [77]. Intriguingly, both in lumen fusion and in cytokinesis, distant portions of the plasma membrane are brought in close proximity by the action of special cytoskeletal structures, which have to disassemble in order to allow subsequent plasma membrane fusion. It will be exciting to integrate the detailed mechanistic knowledge of cytokinesis and of tubulogenesis *in vitro* with the analysis of tube fusion events during organogenesis *in vivo*.

5. Conclusions and outlook

In this review we summarized recent progress, emerging from diverse model systems, in understanding the mechanisms of fusion events in epithelial and endothelial tubular organs. Despite significant progress in the field, several basic questions remain open. It is not understood how fusion

cells recognize each other and establish a single cell-cell contact with the partner cell, whilst avoiding self-contact. A major unresolved point of debate in the field is the question to which extent *de-novo* lumen formation is involved in cell hollowing and which membrane trafficking machineries are employed to build new luminal spaces. Furthermore, how membrane fusion in tubes is spatiotemporally controlled, and what kind of signals (e.g. pH, calcium) are involved, is still mysterious. Visualizing intermediates of the fusion process using high-resolution *in vivo* imaging holds great promise in clarifying these issues. In parallel, systematic identification and tissue-specific manipulation of the proteins involved in tube fusion will help elucidating the underlying molecular mechanisms. Answering basic questions about tip cell behaviour, lumen formation, and conversion of cellular topology in the comparatively simple *Drosophila* tracheal tube fusion model will contribute to a conceptual framework for elucidating similar processes, such as vascular anastomosis and pronephric duct fusion, in more complex vertebrate systems.

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Figure Legends

Figure 1: Anastomoses in the *Drosophila* tracheal system and in the Zebrafish vascular system.

(A) Lateral view of the tracheal system in a *Drosophila* embryo (15 h after egg lay). Each branch contains a stereotyped set of fusion cells (FCs; cyan nuclei) and terminal cells (TCs; red nuclei). The tracheal lumen is shown in white. Abbreviations indicate primary branches: DT, dorsal trunk; LT, lateral trunk; DB, dorsal branches; GB, ganglionic branches; VB, visceral branches.

(B) Schematic representation of one DB anastomosis (region marked by small box in (A)). Each DB contains one FC (cyan) and one TC (red) connected to a stalk cell (SC; grey). Contralateral FCs meet at the dorsal midline.

(C) Confocal image of a FC pair in the DT. Plasma membranes of the two cells are labeled in blue and cyan, respectively. FCs are doughnut-shaped cells lacking autocellular junctions. The arrow marks the luminal axis.

(D) Confocal image of a larval TC expressing cytoplasmic GFP (red). TCs are ramified cells containing seamless lumina.

(E) Confocal image of fused LT branches (large rectangle in (A)). Cells are labeled with a membrane marker. Arrowheads indicate fusion points.

(F) Schematic lateral view of vascular system in larval Zebrafish (36 hpf). Positions of intersegmental vessel (ISV) anastomoses in the trunk are indicated by cyan dots. Note that anastomoses also occur in many other positions not marked in this image.

(G) Confocal image of ISVs in the trunk region of a Zebrafish embryo expressing GFP controlled by an endothelial-specific promoter. Arrowheads indicate anastomosis sites in the dorsal longitudinal anastomotic vessel (DLAV).

Images in panels (F) and (G) were kindly provided by Anna Lenard and Markus Affolter.

Scale bars: (A, D, F), 50 µm; (C, E, G), 10 µm.

Figure 2: Steps of the tracheal tube fusion program.

(A-E) Scheme of *Drosophila* tracheal tube fusion subdivided into five steps as described in the text. A sagittal section through the center of a fusion joint is shown with FCs in cyan, SCs in grey, AJs in light green, FC apical membrane in magenta and the actin/MT track in dark green.

(F) Stills from a time-lapse movie of dorsal branch fusion. Tracheal cells express a plasma membrane marker. FCs were manually highlighted in cyan. Note that the distance (dashed line) between the invading SC luminal tips decreases until the lumen becomes continuous. Time and corresponding steps in panels (A-E) are indicated.

(G) Dorsal branch anastomosis with mosaic labeling. The plasma membrane of the left stalk cell (SC1) is labeled with CFP (yellow), while other tracheal cells are labeled with mCherry (cyan). AJs (α-Catenin-GFP) are labeled in magenta. The FC-FC contact point is marked by an arrowhead. A cross-section at the dashed line (right panel) shows that the SC "finger" is surrounded by the FC. Scale bars, 5 µm.

Figure 3: Cellular machineries involved in tracheal lumen fusion.

Schematic view (sagittal section) of a fusion cell (cyan) and stalk cell (grey) during late-stage tracheal tube fusion (corresponding to Fig. 2D). (a-d) refer to steps of the fusion process as discussed in sections 4.1 through 4.5 in the text.

During repolarization (a), the FC establishes a new apical domain opposite to the tip of the invading stalk cell lumen (left). Apical proteins (aPKC, Crumbs, Sas, PATJ, Rab11, and Sec5) are present at both apical domains (magenta). (b) An F-actin- and microtubule (MT)-containing track is assembled with the help of Formin 3 and is anchored to AJs by the plakin Short stop (Shot). (c) Vesicle trafficking contributes to apical membrane expansion and lumen connection. Exocyst (Sec5), Arl3, and recycling endosomes (Rab11) (yellow) are present on vesicles along the cytoskeletal track and are enriched in subapical regions. Luminal membrane may also be derived from the lateral plasma membrane (blue vesicle), from the Golgi apparatus (orange vesicles), or from unknown trafficking routes (magenta vesicles). Vesicles could be directed via the actin/MT track towards the growing apical domains, or may coalesce to form larger membrane compartments (magenta 'vacuoles'). (d) Connection of the invading stalk cell lumen (left) and the FC central lumen (right) may be achieved

either by direct fusion of the two luminal membranes in a single focal point (‘direct’ model), or by fusion of intermediary membrane compartments (magenta ‘vacuoles’) with each other and with the two apical membranes (‘indirect’ model). Solid lines represent published data, dashed lines are hypothetical.

Figure 1

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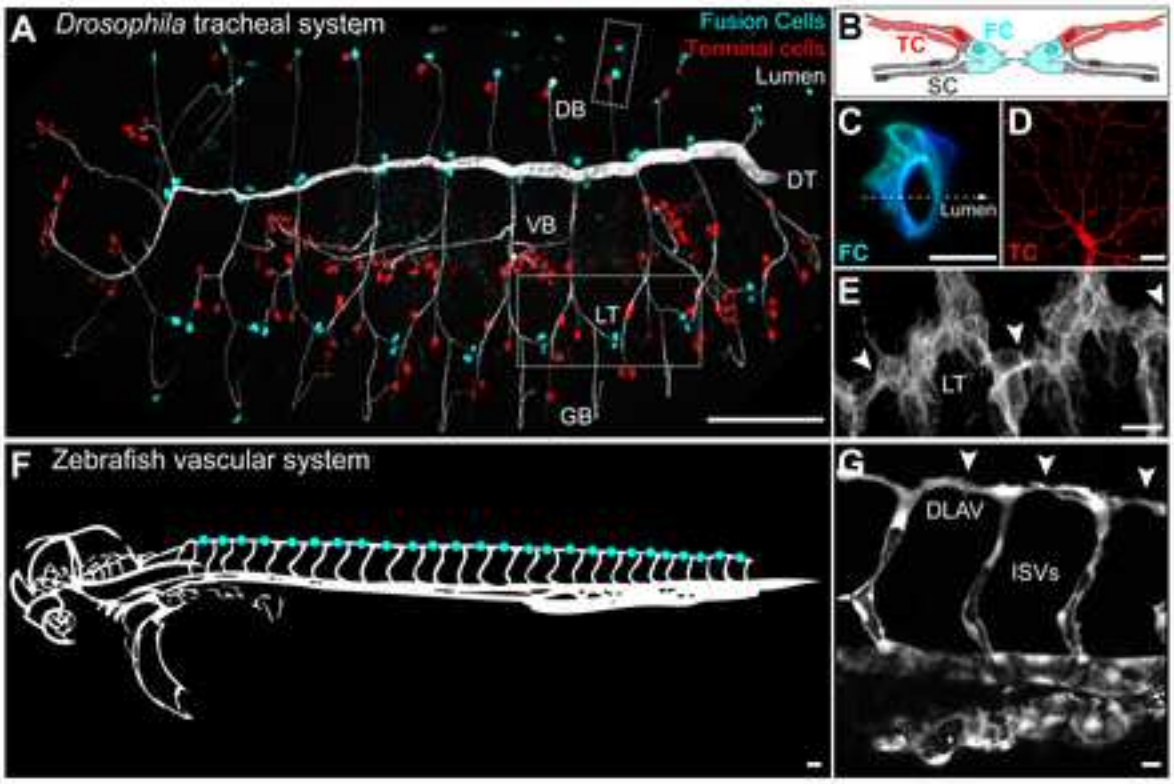

Figure 2

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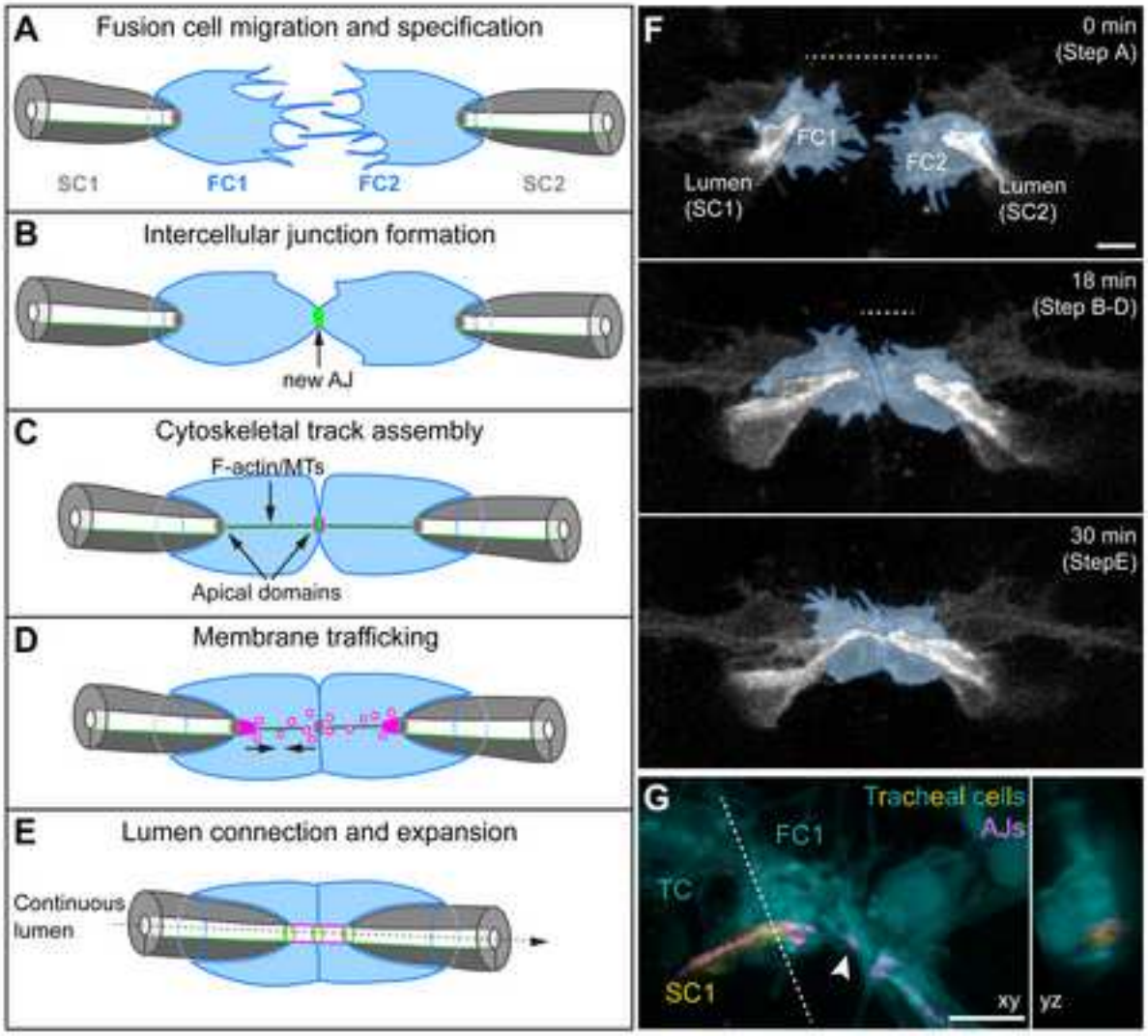

Figure 3
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